Original Article

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Study of Dickkopf-1 (DKK-1) Gene Expression in Hepatocellular Carcinoma Patients

Internal Medicine Section

MONA WATANY[1], REHAB BADAWI[2], WALAA ELKHALAWANY[3], SHERIEF ABD-ELSALAM[4]

ABSTRACT

Introduction: Hepatocellular Carcinoma (HCC) is the sixth most common cancer in the world. Dickkopf -1 (DKK-1) protein is a new biomarker used in conjunction with Alpha Fetoprotein (AFP) to differentiate HCC from "non-malignant" liver disease. DKK-1 is an inhibitor of Wnt/β-catenin signaling pathway which is involved in embryogenesis and has been implicated in tumorigenesis in many tissues.

Aim: To investigate the level of DKK-1 gene expression in the peripheral blood of patients with HCC who had a history of Hepatitis C Virus (HCV) and schistosomal infections.

Materials and Methods: This "cross-sectional" study was carried out in the Tropical Medicine Department of Tanta University Hospital on 50 patients with HCC and 10 healthy volunteers served as control. All patients were tested for HCV antibodies and "anti-schistosomal" antibodies. All groups were tested for DKK-1 gene expression which was measured with quantitative real-time PCR.

Results: DKK-1 gene was over-expressed in HCC patients than in the control group with mean 3.269±4.762 versus 1.00 in controls (p< 0.005). "Over- expression" of DKK-1 was found in: 8/20 of patients with negative serology for both infections (40%; p<0.001), 7/18 of patients with positive anti-HCV antibodies (38.89%; p<0.001) and 11/12 of patients with positive anti-schistosomal antibodies (91.66%; p<0.001). There was no statistically significant correlation between DKK-1 expression and HCV infection (p=0.139) but there was significant correlation between the gene expression and schistosomal infection (p<0.001).

Conclusion: These data suggest the role of DKK-1 over-expression in HCC development in patients with combined HCV and schistosomal infections and that induction of the Wnt pathway or using DKK-1 antagonist may represent a key advance in the area of genetic prevention of HCC in these "high-risk" patients.

Keywords: Biomarker, Hepatitis C virus, Tumorigenesis

INTRODUCTION

HCC is the sixth most common cancer in the world, but the second most common cause of cancer death [1]. In Singapore, from 2009 to 2013, it positioned as the third and fourth most basic reason for malignancy passing amongst guys and females individually [2] while, in Egypt, HCC is the fourth most common cancer and is the second cause of cancer mortality in both sexes [3]. Unfortunately, HCC development is symptomless in early stages of the illness when current curative therapies are effective. Many tools for HCC screening was used including serological and imaging examinations [4]. The most widely-used HCC biomarker is the serum α-Fetoprotein (AFP) [5], and many physicians currently use AFP in clinical practice to diagnose HCC [6]. However, the current Western guidelines [7] have excluded AFP measurement for the diagnosis of HCC, because of its limited accuracy in detecting HCC, with a sensitivity of about 60% at a cut-off value of 20 ng/mL [8] and low specificity [9]. Novel and reliable diagnostic biomarkers are required to complement AFP [10]. To complement the limitations of AFP, the combined measurement of AFP and DKK-1 have been used.

HCC manifestation diversity is found in the genetic and epigenetic alterations [11]. One of these, alterations is Wnt/β-catenin signaling which has been proved to play a critical role in HCC development [12]. Hence, to know the Wnt/β-catenin signaling pathway is important to identify potential endogenous molecular targets for drugs.

Secreted Wnt antagonists play important roles in regulating Wnt/β-catenin signaling [13], of which, dickkopf-1 (DKK-1), a prototypical member of a secreted protein family is a known potent antagonist of Wnt/β-catenin signaling [14]. DKK-1 acts as an inhibitory ligand of the Low-Density Lipoprotein (LDL) receptor-related protein 5/6 co-receptors. It blocks their interaction with Wnt, thus, causing in β-catenin degradation [15].

DKK-1 is down-regulated in human colon tumours; hence, it may act as a tumour suppressor. High DKK-1 expression indicated favourable responses to chemotherapy in brain tumours [16] whereas; over-expression of DKK-1 was found in human hepatoblastomas, Wilms' tumours and multiple myelomas [17].

So, DKK-1 was recently reported as a promising biomarker for HCC, even in AFP-negative patients, and a combination of AFP and DKK-1 measurement showed an improved diagnostic accuracy among HBV infected patients [10].

MATERIALS AND METHODS

This cross-sectional study was carried out in the tropical medicine department inpatient and outpatient clinic, Tanta University Hospital, Egypt in the period from April 2015 to April 2016 and included 50 patients with HCC and 10 healthy subjects as control. Informed written consents were obtained from all participants and the study was approved by the Local Ethics Committee of Tanta Faculty of Medicine, Tanta, Egypt.

All patients presented to our department during this period, meeting the inclusion criteria, were included in the study.

Diagnosis of HCC was made on the following criteria: 1) pathological HCC diagnosis for patients who underwent surgical resection or percutaneous biopsy; or 2) clinical and radiological HCC diagnosis for patients without available HCC tissue specimens, based on the guidelines of the American Association for the Study of Liver Diseases. HCC stage was clinically defined according to the Barcelona Clinic Liver Cancer (BCLC) staging system.

The exclusion criteria were- previous history of HCC ablation,

pathological or radiological evidence of mixed HCC-cholangio cellular carcinoma, extrahepatic malignancy.

The total number of patients included were 60, 50 of them were HCC patients and 10 were healthy controls. They were divided in the following groups:

Group I: 20 patients diagnosed with HCC with negative serological markers for HCV and schistosomal infection.

Group II: 18 patients diagnosed with HCC with positive HCV antibodies.

Group III: 12 patients diagnosed with HCC with positive HCV antibodies and positive anti-schistosomal antibodies.

Control group: 10 healthy subjects.

All patients were subjected to the following: history taking, thorough clinical examination, liver function tests, complete blood picture, kidney function tests, blood sugar, and abdominal ultrasonography.

Blood samples: A 2 ml of peripheral blood was collected in EDTA vacutainer for genetic study, 5 ml was collected in serum separator tubes for testing for HCV-Ab and schistosomal-Ab, the tubes was centrifuged and sera were preserved at -70°C till testing.

HCV antibody was measured using commercially available kit (ORTHO® HCV Version 3.0 ELISA Test System, USA) according to the manufacturer's instructions. Bilharzial-Ab was assayed using commercially available kit (Accu Diag™ SchistosomaIgG ELISA Kit, USA) according to manufacture's instructions.

DKK-1 gene expression was measured with quantitative real-time PCR analysis from the peripheral blood of 50 patients with HCC and 10 controls.

RNA was extracted from 1 ml blood with Qiamp® RNA Blood Mini Kit (Qiagen GmbH, Germany), according to the manufacturer's instructions.

Total RNA from each sample was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions, using Biometra® thermal cycler (Germany). TaqMan real-time quantitative PCR amplification reactions were carried out with step one® Real–Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). The reaction mixture contained about 30ngcDNA, 1µLTaqman assay® and 10µL 2×TaqMan Buffer® (Applied Biosystems, USA) in a total reaction volume of 20 µL under standard conditions (initial setup 2 minutes – 50°C, 10 minutes – 95°C, 40 cycle of (denaturation 15 seconds – 95°C, annealing 1 minutes – 60°C).

GAPDH was used as an internal control gene. All reactions were executed in duplicate. In the case of negative control, cDNA was not added.

The relative concentrations of DKK-1 mRNA were calculated on basis of Cycle Threshold (CT), corrected by GAPDH expression with the comparative method formula $2^{-\Delta Ct}$ where {Δ Ct = Ct(DKK-1) – Ct(GAPDH)}.

STATISTICAL ANALYSIS

Results were statistically analysed by SPSS statistical package version 20 (SPSS Inc. Released 2011. IBM SPSS statistics for windows, version 20.0, Armnok, NY: IBM Corp.).

Kruskal Wallis test was used for comparison of quantitative variables between more than two groups of not normal distributed data with Tukey's test as post hoc test. Spearman correlation was not used for normally distributed ones. The p-value of < 0.05 was considered statistically significant.

RESULTS

Data were presented as the fold change in gene expression normalized to the endogenous reference gene and relative to the healthy control group. There was statistically significant difference between the 4 studied groups regarding the mean gene expression (p value <0.001) as Group III showed a significantly higher level (8.31±7.77) than Group I (1.68±p 1.15, p<0.001), Group II (1.66±1.04, p<0.001) and control Group (1.00±0.00, p<0.001).

Overexpression of DKK-1 was found in: 8/20 of patients in Group I (40%), 7/18 of Group II (38.89%) and 11/12 0f Group III (91.66%) as shown in [Table/Fig-1].

	No of cases with over-expression	p-value
Group I	8/20 (40%)	<0.001
Group II	7/18 (38.89%)	<0.001
Group III	11/12 (91.66%)	<0.001

[Table/Fig-1]: Number of cases with over-expression of DKK-1gene.

DISCUSSION

HCC is one of the cancers with a high rate of dysregulation in the Wnt/β-catenin pathway [18]. Dysregulation of Wnt/β-catenin signaling pathway is a hallmark of major gastrointestinal cancers including HCC [19]. The development of successfully targeted therapies for HCC is dependent on the identification of signaling pathways used by tumour cells to proliferate, invade or metastasize during the progress of tumour growth.

Rarely DKK-1 is expressed in normal adult tissues with the exception of placenta and embryonic tissues [20], many tumours show a wide range of DKK-1 expression at various phases of tumourigenesis including prostate, breast, colorectal, esophageal, lung, and Multiple Myeloma (MM) [20,21]. Prostate cancers usually express lower DKK-1 levels compared to normal prostate tissue [22].

Our study showed that DKK-1 gene expression was significantly elevated in HCC patients than the control group; p< 0.005. There is almost in agreement with all published data regarding DKK-1 over-expression in HCC patients.

Our results were in concordance with Yu et al., and with Tung et al., who reported elevated serum DKK-1 protein level in HCC patients [23,24]. Also, Shen et al., reported elevated both serum level and DKK-1 mRNA in liver tissue samples from patients with HCC even more than AFP mRNA [10].

Yu et al., studied DKK-1 expression in HCC cell lines using qRT-PCR, Western blotting, immunofluorescence and tissue microarrays they reported the correlation between DKK-1 over-expression and β-catenin cytoplasmic/nuclear accumulation in clinical HCC samples and its association with poor prognosis [23].

Supporting these observations Kim et al., studied DKK-1 gene expression in Hep3B, Huh, 7 HCC cell lines and 293 cells which served as control; they showed high DKK-1 mRNA expression and protein secretion in the culture media of HCC cell lines in comparison to the control [25].

Tung and Ng studied serum level of DKK-1 protein in HCC patients and reported the improvement of diagnostic accuracy the DKK-1 adds to AFP [26].

Huang et al., documented the role of DKK-1 in HCC cell invasion and metastasis and declared that DKK-1 over expression may be a potential molecular therapeutic target for liver cancer [27].

In a meta- analysis study Liu et al., studied DKK-1 gene expression in several solid tumours, they found DKK-1 was significantly over-expressed in HCC and related to poor disease free survival [28].

Tung et al., found that both DKK-1 transcript and serum protein were up regulated in a stepwise manner in human HCCs and its transcript levels were associated with more aggressive tumour behaviour [24].

There was no statistically significant correlation between HCV infection and level of DKK-1 expression (p= 0.139), this was in concordance with Liu et al., who reported that HCV core protein

activates Wnt/β-catenin signaling with over-expression of canonical Wnt ligands, such as Wnt2, Wnt3, Wnt3a, Wnt8b, Wnt10a, Wnt10b, frizzled receptors Fzd1, 2, 5, 6, 7, 9, and LRP5/6 co-receptors and moderately repression of Wnt antagonists like SFRP3, 5 and DKK-1 [29]. Also, Fouad et al., found no statistically difference between DKK-1 serum level in HCV patients and healthy controls [30]. More recently, Wnt signaling has been shown to participate in human fibrosing diseases, such as pulmonary fibrosis, renal fibrosis, and liver fibrosis [31].

LIMITATION

The limitation of this study is the relatively small number of patients. Larger multi-centeric studies are needed to confirm the findings of this study.

CONCLUSION

To our knowledge this is the first study to investigate DKK-1 expression in HCC patients with previous schistosomal infections. A statistically significant correlation between schistosomal infection and DKK-1 expression ($p<0.001$) has been found. Liver fibrosis is one of the most common wound-healing responses to chronic liver injuries. Schistosomal infection is the most common cause of liver fibrosis in Egypt. Wnt genes and their translated proteins proved to participate in the regulation of cell proliferation, polarity and differentiation.

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PARTICULARS OF CONTRIBUTORS:

1. Department of Clinical Pathology, Tanta University Hospital, Tanta, Egypt.
2. Department of Tropical Medicine, Tanta University Hospital, Tanta, Egypt.
3. Department of Tropical Medicine, Tanta University Hospital, Tanta, Egypt.
4. Department of Tropical Medicine, Tanta University Hospital, Tanta, Egypt.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Sherief Abd-Elsalam,
Department of Tropical Medicine, Tanta University Hospital, Tanta, Egypt.
E-mail: sherif_tropical@yahoo.com

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